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ISOLATED AND PURIFIED DNA SEQUENCE CODING ANTIGEN EXPRESSED BY TUMOR CELLS AND RECOGNIZED BY CYTOTOXIC T CELLS, AND USES THEREOF

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This application is a continuation-in-part of Serial Number 705,702, filed May 23, 1991, and now abandoned.

FIELD OF THE INVENTION

This invention relates in general to the field of immunogenetics as applied to the study of oncology. More specifically, it relates to the study and analysis of mechanisms by which tumors are recognized by the organism's immune system such as through the expression of so-called tumor rejection antigens.

BACKGROUND AND PRIOR ART

The study of the recognition or lack of recognition of cancer cells by a host organism has proceeded in many different directions. Understanding of the field presumes some understanding of both basic immunology and oncology.

Early research on mouse tumors revealed that these displayed molecules which led to rejection of tumor cells when transplanted into syngeneic animals. These molecules are "recognized" by T-cells in the recipient animal, and provoke a T-cell response with lysis of the transplanted cells. This evidence was first obtained with tumors induced in vitro by chemical carcinogens, such as methylcholanthrene. The antigens expressed by the tumors and which elicited the T-cell response were found to be different for each tumor. See Prehn, et al., J. Natl. Canc. Inst. 18: 769-778 (1957); Klein et al., Cancer Res. 20: 1561-1572 (1960); Gross, Cancer Res.

3: 326-333 (1943), Basombrio, Cancer Res. 30: 2458-2462 (1970) for general teachings on inducing tumors with chemical carcinogens and differences in cell surface antigens. This class of antigens has come to be known as "tumor specific transplantation antigens" or "TSTAs". Following the observation of such antigens when induced by chemical carcinogens, similar results were obtained when tumors were induced in vitro via ultraviolet radiation. See Kripke, J. Natl, Canc. Inst. 53: 333-1336 (1974).

While T-cell mediated immune responses were observed for the types of tumor described supra, spontaneous tumors were thought to be generally non-immunogenic. These were therefore believed to be free of antigens which provoked a response to the tumor in the tumor carrying subject. See Hewitt, et al., Brit. J. Cancer 33: 241-259 (1976).

The family of tum^- antigens are immunogenic variants obtained by mutagenesis of mouse tumor cells, as described by Boon et al., J. Exp. Med. 152: 1184-1193 (1980), the disclosure of which is incorporated by reference. To elaborate, tum^- antigens are obtained by mutating tumor cells which do generate an immune response in syngeneic mice, known as tum^+ antigens. When these cells are mutagenized, they are rejected by syngeneic mice, and fail to form tumors (thus "tum^-"). See Boon et al., Proc. Natl. Acad. Sci. USA 74: 272 (1977), the disclosure of which is incorporated by reference. Many tumor types have been shown to

exhibit this phenomenon. See, e.g., Frost et al., Cancer Res. 43: 125 (1983).

It appears that tum^- variants fail to form progressive tumors because they elicit an immune rejection process. The evidence in favor of this hypothesis includes the ability of "tum^-" variants of tumors, i.e., those which do not normally form tumors, to do so in mice with immune systems suppressed by sublethal irradiation, Van Pel et al., Proc. Natl, Acad. Sci. USA 76: 5282-5285 (1979); and the observation that intraperitoneally injected tum^- cells of mastocytoma P815 multiply exponentially for 12-15 days, and then are eliminated in only a few days in the midst of an influx of lymphocytes and macrophages (Uyttenhove et al., J. Exp. Med. 152: 1175-1183 (1980)). Further evidence includes the observation that mice acquire an immune memory which permits them to resist subsequent challenge to the same tum^- variant, even when immunosuppressive amounts of radiation are administered with the following challenge of cells (Boon et al., Proc. Natl, Acad. Sci. USA 74: 272-275 (1977); Van Pel et al., supra; Uyttenhove et al., supra).

Later research found, that when spontaneous tumors were subjected to mutagenesis, immunogenic variants were produced which did generate a response. Indeed, these variants were able to elicit an immune protective response against the original tumor. See Van Pel et al., J. Exp. Med. 157: 1992-2001 (1983). Thus, it

has been shown that it is possible to generate expression of a so-called tumor rejection antigen in a tumor which is a target for syngeneic rejection response. Similar results have been obtained when foreign genes have been transfected into spontaneous tumors. See Fearson et al., Cancer Res. 48: 2975-1980 (1988) in this regard.

A class of antigens has been recognized which are presented on the surface of tumor cells and are recognized by cytotoxic T cells, leading to lysis. This class of antigens will be referred to as "tumor rejection antigens" or "TRAs" hereafter. TRAs do not elicit antibody responses. As a result, they cannot be identified and/or characterized in a manner involving immune precipitation. To the extent these antigens have been studied, this has resulted from cytolytic T cell characterization studies, i.e., the study of the identification of the antigen by a particular cytolytic T cell ("CTL" hereafter) subset. The subset proliferates upon recognition of the tumor rejection antigen, and the cells presenting the antigen are lysed. Characterization studies have identified and lysed CTL clones which specifically lyse cells expressing the antigens. Examples of this work may be found in Levy et al., Adv. Cancer Res. 24: 1-59 (1977); Boon et al., J. Exp. Med. 152: 1184-1193 (1980); Brunner et al., J. Immunol. 124: 1627-1634 (1980); Maryanski et all, Eur. J. Immunol. 124: 1627-1634 (1980); Maryanski et al., Eur. J. Immunol. 12: 406-412 (1982); Palladino et al., Canc. Res. 47: 5074-5079 (1987). This type of analysis is required for other types of antigens recognized by CTLs, including minor

histocompatibility antigens, the male specific H-Y antigens, and a class of antigens, referred to as "tum-" antigens, and discussed herein.

A tumor exemplary of the subject matter described supra is known as P815. See DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988); Szikora et al., EMBO J 9: 1041-1050 (1990), and Sibille et al., J. Exp. Med. 172: 35-45 (1990), the disclosures of which are incorporated by reference. The P815 tumor is a mastocytoma, induced in a DBA/2 mouse with methylcholanthrene and cultured as an in vitro tumor and cell line. The P815 line has generated many tum^- variants following mutagenesis, including variants referred to as P91A (DePlaen, supra), 35B (Szikora, supra), and P198 (Sibille, supra). In contrast to tumor rejection antigens - and this is a key distinction - the tum^- antigens are only present on mutagenized cells. Tumor rejection antigens are present on cells of a given tumor prior to mutagenesis. Hence, with reference to the literature, a cell line can be tum^+, such as the line referred to as "P1", and can be provoked to produce tum^- variants. Since the tum^- phenotype differs from that of the parent cell line, one expects a difference in the DNA of tum^- cell lines as compared to their tum^+ parental lines, and this difference can be exploited to locate the gene of interest in tum^- cells. As a result, it was found that genes of tum^- variants such as P91A, 35B and P198 differ from their normal alleles by point mutations in the coding regions of the gene. See Szikora and Sibille, supra, and

Lurquin et al., Cell 58: 293-303 (1989). This has proved not to be the case with the TRAs of the invention. These papers also demonstrated that peptides derived from the tum^- antigen are presented by the L^d molecule for recognition by CTLs.

It has now been found, surprisingly, that the genes for tumor rejection antigens or "TRAs", which are the subject of this application, are not mutated genes; but occur in both tumorigenic and non-tumorigenic cells. Since the gene is not a mutation, one would expect a similar response against normal cells, leading to autoimmune responses and the consequences thereof. Again, surprisingly in view of what is known about tum^- antigens and expression, this does not occur.

The lack of any difference between the gene for tumor rejection antigens in normal and carcinogenic cells was not observed until a gene was actually isolated and cloned, making it all the more surprising that the gene could be found.

The gene is useful as a source for the isolated and purified tumor rejection antigen itself, which can be used as an agent for treating the cancer for which the antigen is a "marker". It is known, for example, that tum^- cells can be used to generate CTLs which lyse cells presenting different tum^- antigens as well as tum^+ cells. See, e.g., Maryanski et al., Eur. J. Immunol 12: 401 (1982); and Van den Eynde et al., Modern Trends in Leukemia IX (June 1990), the disclosures of which are incorporated by reference. The tumor rejection antigen may be expressed in cells

transformed by the gene, and then used to generate an immune response against a tumor of interest.

In the parallel case of human neoplasms, it has been observed that autologous mixed lymphocyte-tumor cell cultures ("MLTC" hereafter) frequently generate responder lymphocytes which lyse autologous tumor cells and do not lyse natural killer targets, autologous EBV-transformed B cells, or autologous fibroblasts (see Anichini et al., Immunol. Today 8: 385-389 (1987)). This response has been particularly well studied for melanomas, and MLTC have been carried out either with peripheral blood cells or with tumor infiltrating lymphocytes. Examples of the literature in this area including Knuth et al., Proc. Natl. Acad. Sci. USA 86: 2804-2802 (1984); Mukherji et al., J. Exp. Med. 158: 240 (1983); Hérin et all, Int. J. Canc. 39: 390-396 (1987); Topalian et al, J. Clin. Oncol 6: 839-853 (1988). Stable cytotoxic T cell clones ("CTLs" hereafter) have been derived from MLTC responder cells, and these clones are specific for the tumor cells. See Mukherji et al., supra, Hérin et all, supra, Knuth et al., supra. The antigens recognized on tumor cells by these autologous CTLs do not appear to represent a culturel artifact, since they are found on fresh tumor cells. Topalian et al., supra; Degiovanni et al., Eur. J. Immunol. 20: 1865-1868 (1990). These observations, coupled with the techniques used herein to isolate the TRA for specific murine tumors, have led to the isolation of nucleic acid sequences coding for tumor rejection antigens on human tumors, melanoma in particular. It is now possible to isolate the nucleic acid

sequences for tumor rejection antigens characteristic of the particular tumor, with ramifications that are described infra. These isolated nucleic acid sequences for human tumor rejection antigen and applications thereof, as described infra, are also the subject of this invention.

These and various other aspects of the invention are elaborated upon in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts detection of transfectants expressing antigen P815A.

Figure 2 shows the sensitivity of clones P1.HTR, PO.HTR, genomic transfectant P1A.T2 and cosmid transfectant P1A.TC3.1 to lysis by various CTLs, as determined by chromium release.

Figure 3 is a restriction map of cosmid C1A.3.1.

Figure 4 shows Northern Blot analysis of expression of gene P1A.

Figure 5 sets forth the structure of gene P1A with its restriction sites.

Figures 6a, 6b, 6c and 6d present the nucleotide and amino acid sequence for the three exons and 5′ region of gene P1A. This information is also presented in sequence id no: 1, sequence id no: 2, and sequence id no: 3. The full cDNA for the sequence is available from EMBL/GenBank (accession number M36387). The

complete sequence between 0.7 and 5 of Figure 5 is also deposited with EMBL/GenBank (accession number M36386).

Figure 7 shows the results obtained when cells were transfected with the gene from P1A, either isolated from P815 or normal cells and then tested with CTL lysis.

Figure 8 shows lytic studies using mast cell line L138. 8A.

Figure 9 presents the entire sequence of P1A gene, with exons presented in capital letters.

Figure 10 sets forth the amino acid sequence of antigenic peptides for the P1A TRA. The sequence is for cells which are A^+, B^+, i.e., express both the A and B antigens. The peptide corresponding to phenotype A^- B^+ is identical, except for a change from valine to alanine at position 18.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

EXAMPLE 1

In order to locate the gene coding for antigen P815A, gene transfection was used. This approach requires both a source of the gene, and a recipient cell line. Highly transfectable cell line P1.HTR was the starting material for the recipient, but it could not be used without further treatment, as it expresses antigen A, one of four recognized P815 tumor antigens. See Van Pel et al.,

Molecular Genetics 11: 467-475 (1985). Thus, screening experiments were carried out to isolate cell lines which did not express the antigen and which nonetheless possessed P1.HTR's desirable qualities.

To do this, P1.HTR was screened with CTLs which were specific for each of tumor antigens A, B, C and D. Such CTLs are described by Uyttenhove et al., J. Exp. Med. 157: 1040-1052 (1983).

To carry out the screening, the CTLs were incubated with 2000 ^{51}Cr labelled cells at various ratios of effector/target cells, in 96 well conical microplates in final volume of 200 ul. After 4 hours of incubation at 37°C, chromium release was measured. Additional information on the CTL assay and screening protocol, in general may be found in Boon et al., J. Exp. Med. 152: 1184-1193 (1980), and Maryanski et al., Eur. J. Immunol. 12: 406-412 (1982).

When these screenings were carried out, a cell line variant was found which expressed neither antigen A or B. Additional screenings then yielded a variant which also lacked antigen C. Please see figure 2 for a summary of the results of these screening. The variant PO.HTR is negative for antigens A, B and C, and was therefore chosen for the transfection experiments.

The cell line PO.HTR has been deposited in accordance with the Budapest Treaty at the Institute Pasteur Collection Nationale De Cultures De Microorganismes, 28, Rue de Docteur Roux, 75724 Paris France, and has accession number I-1117.

This methodology is adaptable to secure other cell lines which are variants of a cell type which normally expresses at least one of the four recognized P815 tumor antigens, i.e., antigens A, B, C and D, where the variants express none of antigens A, B and C. P1.HTR is a mastocytoma cell line, so it will be seen that the protocol enables the isolation of biologically pure mastocytoma cell lines which express none of P815 antigens A, B and C, but which are highly transfectable. Other tumor types may also be screened in this fashion to secure desired, biologically pure cell lines. The resulting cell lines should be at least as transfectable with foreign DNA as is P1.HTR, and should not express P815 antigens A, B and C.

EXAMPLE 2

Previous work reported by DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988) the disclosure of which is incorporated by reference had shown the efficacy of using cosmid library transfection to recover genes coding for tum^- antigens.

The entire plasmid and genomic DNA of P1.HTR was prepared, following Wölfel et al., Immunogenetics 26: 178-187 (1987). The transfection procedure followed Corsaro et al., Somatic Cell Molec. Genet 7: 603-616 (1981), with some modification. Briefly, 60 μg of cellular DNA and 3 μg of DNA of plasmid pHMR272, described by Bernard et al., Exp. Cell. Biol. 158: 237-243 (1985) were mixed. This plasmid converts hygromycin resistance upon recipient cells,

and therefore provides a convenient way to screen for transfectants. The mixed DNA was combined with 940 ul of 1 mM Tris-HCl (pH 7.5), 0.1 mM EDTA; and 310 ul 1M $CaCl_2$. The solution was added slowly, and under constant agitation to 1.25 ml of 50 mM Hepes, 280 mM NaCl, 1.5 mM Na_2HPO_4, adjusted to pH 7.1 with NaOH. Calcium phosphate - DNA precipitates were allowed to form for 30-45 minutes at room temperature. Following this, fifteen groups of PO.HTR cells ($5x10^6$) per group were centrifuged for 10 minutes at 400 g. Supernatants were removed, and pellets were resuspended directly into the medium containing the DNA precipitates. This mixture was incubated for 20 minutes at 37°C, after which it was added to an 80 cm_2 tissue culture flask containing 22.5 ml DMEM, supplemented with 10% fetal calf serum. After 24 hours, medium was replaced. Forty-eight hours after transfection, cells were collected and counted. Transfected cells were selected in mass culture using culture medium supplemented with hygromycin B (350 ug/ml). This treatment selected cells for hygromycin resistance.

For each group, two flasks were prepared, each containing $8x10^6$ cells in 40 ml of medium. In order to estimate the number of transfectants, $1x10^6$ cells from each group were plated in 5 ml DMEM with 10% fetal calf serum (FCS), 0.4% bactoagar, and 300 ug/ml hygromycin B. The colonies were then counted 12 days later. Two independent determinations were carried out and the average taken. This was multiplied by 5 to estimate the number of transfectants

in the corresponding group. Correction had to be made for the cloning efficiency of P815 cells, known to be about 0.3.

EXAMPLE 3

Eight days after transfection as described in example 2, supra, antibiotic resistant transfectants were separated from dead cells, using density centrifugation with Ficoll-Paque. These cells were maintained in non-selective medium for 1 or 2 days. The cells were plated in 96 well microplates (round bottom), at 30 cells/microwell in 200 ul of culture medium. Anywhere from 100-400 microwells were prepared, depending on the number of transfectants prepared. Agar colony tests gave estimates of 500-3000. After 5 days, wells containing about $6x10^4$ replica plates were prepared by transferring 1/10 of the wells to microplates which were then incubated at 30°C. One day later, master plates were centrifuged, medium removed, and 750 CTLs against P815 antigen A (CTL-P1:5) were added to each well together with 10^6 irradiated syngeneic feeder spleen cells in CTL culture medium containing 40 U/ml recombinant human IL-2, and HAT medium to kill stimulator cells. Six days later, plates were examined visually to identify wells where CTLs had proliferated. Where plates showed proliferating microcultures, aliquots of 100 ul of the wells were transferred to another plate containing ^{51}Cr labeled P1.HTR target cells ($2x10^3$ - $4x10^3$ per well), and chromium release was measured after 4 hours. Replica microcultures corresponding to those

showing high CTL activity were expanded and cloned by limited dilution in DMEM with 10% FCS. Five days later, about 200 clones were collected and screened with the CTL.P1:5 cell line, described supra, in a visual lysis assay. See figure 1A for these results.

In these experiments, three of the fifteen groups of transfectants yielded a few positive microcultures. These microcultures were tested for lytic activity against P1.HTR, as described supra. Most of the microcultures where proliferation was observed showed lytic activity. This activity was well above background, as shown in figure 1B. This figure summarizes data wherein two groups of cells (groups "5" and "14"), 400 and 300 microwells were seeded with 30 hygromycin resistant transfected cells. Amplification and duplication of the microcultures was followed by addition of anti-A CTL P1:5. Six days later, lytic activity against P1.HTR was tested. In the figure, each point represents lytic activity of a single microculture.

Duplicate microcultures corresponding to several positive wells were subcloned, and more than 1% of the subclones were found to be lysed by anti-A CTL. Thus, three transfectants expressing P815A were obtained from 33,000 hygromycin resistant transfectants. One of these lines, referred to hereafter as P1A.T2 was tested further.

The relevant antigen profile of P1A.T2 is shown in figure 2, this being obtained via anti-CTL assays of the type described supra.

EXAMPLE 4

The CTL assays carried out for P1A.T2 demonstrated that it expressed antigen A ("P815A"), and therefore had received the gene from P1.HTR. To that end, this cell line was used as a source for the gene for the antigen in the following experiments.

Prior work had shown that genes coding for tum^- antigens could be recovered directly from transfectants obtained with a cosmid library. See DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988). This procedure was followed for recovery of the P815 gene.

Total genomic DNA of P1A.T2 was partially digested with restriction endonuclease Sau 3A1, and fractionated by NaCl density gradient ultracentrifugation to enrich for 35-50 kb DNA fragments, following Grosveld et al., Gene 10: 6715-6732 (1982). These fragments were ligated to cosmid arms of C2RB, described by Bates et al., Gene 26: 137-146 (1983). These cosmid arms had been obtained by cleavage with SmaI and treatment with calf intestinal phosphatase, followed by digestion with BamHI. Ligated DNA was packaged into lambda phage components, and titrated on E. coli ED 8767, following Grosveld et al., supra. Approximately $9x10^5$ ampicillin resistant colonies were obtained per microgram of DNA insert.

The cosmid groups were amplified by mixing 30,000 independent cosmids with 2 ml of ED 8767 in 10 mM $MgCl_2$, incubated 20 minutes at 37°C, diluted with 20 ml of Luria Bertani medium, followed by

incubation for one hour. This suspension was titrated and used to inoculate 1 liter of LB medium in the presence of ampicillin (50 ug/ml). At a bacterial concentration of $2x10^8$ cells/ml (OD_{600}=0.8), a 10 ml aliquot was frozen, and 200 ug/ml chloramphenicol was added to the culture for overnight incubation. Total cosmid DNA was isolated by alkaline lysis procedure, and purified on CsCl gradient.

In these experiments, a library of 650,000 cosmids was prepared. The amplification protocol involved the use of 21 groups of approximately 30,000 cosmids.

EXAMPLE 5

Using the twenty-one groups of cosmids alluded to supra, (60 ug) and 4 ug of pHMR272, described supra, groups of $5x10^6$ PO.HTR cells were used as transfectant hosts. Transfection was carried out in the same manner as described in the preceding experiments. An average of 3000 transfectants per group were tested for antigen presentation, again using CTL assays as described. One groups of cosmids repeatedly yielded positive transfectants, at a frequency of about 1/5,000 drug resistant transfectants. The transfectants, as with P1A.T2, also showed expression of both antigen A and B. The pattern of expression of transfectant p1A.TC3.1 is shown in figure 2.

EXAMPLE 6

As indicated in Example 5, supra, three dependent cosmid transfected cells expressed P815A antigen. The DNA of these transfectants was isolated and packaged directly with lambda phage extracts, following DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988). The resulting product was titrated on E. coli ED 8767 with ampicillin selection, as in Example 5. Similarly, transfection and amplification of the cosmids followed example 5, again using PO.HTR.

High frequencies of transfection were observed, as described in Table 1, which follows:

Table 1. Transfer of the expression of antigen P815A by cosmids obtained by direct packaging

Transfectant obtained with the cosmid library	No. of cosmids obtained by direct packaging of 0.5 µg of DNA	No. of transfectants expressing P815A / no. of $Hm^{r}B^{r}$ transfectants
TC3.1	32	87/192
TC3.2	32000	49/384
TC3.3	44	25/72

The cosmids were analyzed with restriction enzymes and it was found that directly packaged transfectant P1A.TC3.1 contained 32 cosmids, 7 of which were different. Each of these 7 cosmids was transfected into PO.HTR, in the manner described supra, and again, following the protocols described above, transfectants were studied for expression of P815A. Four of the cosmid transfectants showed P815A expression and, as with all experiments described herein, P815B was co-expressed.

Of the four cosmids showing expression of the two antigens, cosmid C1A.3.1 was only 16.7 kilobases long, and was selected for further analysis as described infra.

The cosmid C1A.3.1 was subjected to restriction endonuclease analysis, yielding the map shown in Figure 3.

All EcoRI fragments were transfected, again using the above described protocols, and only the 7.4 kilobase fragment produced a transfectant that anti-A CTLs could lyse. Similar experiments were carried out on the PstI fragments, and only a 4.1 kb fragment fully contained within the 7.4 kb EcoRI fragment produced lysable transfectants.

This fragment (i.e., the 4.1 kb PstI fragment), was digested with SmaI, giving a 2.3 kb fragment which also passed expression of antigens A and B to the host cell. Finally, a fragment 900 bases long, secured with SmaI/XbaI, also transferred expression of the two antigens.

EXAMPLE 7

The 900 base fragment described above was used as a probe to isolate the gene for P815 from parent cell line P1.HTR. To accomplish this, total cellular RNA was first isolated using the guanidine-isothiocyanate procedure of Davis et al., Basic Methods In Molecular Biology (Elseview Science Publishing Co, New York) (1986). The same reference was the source of the method used to isolate and purify polyA$^+$ mRNA using oligoT on cellulose column chromatography.

Samples were then subjected to Northern Blot analysis. RNA samples were fractionated on 1% agarose gels containing 0.66 M formaldehyde. The gels were treated with 10xSSC (SSC: 0.15 M NaCl; 0.015 M sodium citrate, pH 7.0) for 30 minutes before overnight blotting on nitrocellulose membranes. These were baked for two hours at 80°C, after which the membranes were prehybridized for 15 minutes at 60°C in a solution containing 10% dextran sulfate, 1% SDS and 1M NaCl. Hybridization was then carried out using denatured probe (the 900 base fragment), together with 100 ug/ml salmon sperm DNA.

When this protocol was carried out using P1.HTR poly A$^+$ RNA, a band of 1.2 kb and two fainter bands were identified, as shown in Figure 4, lane 1 (6 ug of the RNA).

The same probe was used to screen cDNA of P1, prepared from poly-A$^+$ RNA from the cell line. This yielded a 1kb insert,

suggesting a missing 5′ end. The Northern blots for the cDNA are not shown.

Hybridization experiments in each case were carried out overnight at 60°C. The blots were washed twice at room temperature with 2xSSC and twice at 60°C with 2xSSC supplemented with 1% SDS.

The foregoing experiments delineated the DNA expressing the P815A antigen sufficiently to allow sequencing, using the well known Sanger dideoxy chain termination method. In order to do this, clones were generated using a variety of restriction endonucleases and synthetic oligonucleotide primers. The results for exons of the gene are set forth in figure 6 and sequence id no: 1, with corresponding amino acids presented below.

EXAMPLE 8

Given the sequencing information described supra, it was possible to further study the cDNA hybridization fragment (1 kb) to determine if the hypothesis that a 5′ fragment had been lost was correct. To do this, a primer corresponding to positions 320-303 was used. Amplification was carried out using a 3′ primer corresponding to positions 286-266 and 5′ primers described by Frohman et al. Proc Natl. Acad. Sci. USA 85: 8998-9002 (1988). A band of expected size (270 bases), was found, which hybridized to the probe described supra when Southern blotting was carried out, proving the assertion was correct. Following cloning into M13 tg 130 and tg 131, the small, 270 base pair band was sequenced.

EXAMPLE 9

Following the procurement of the sequences described in Examples 7 and 8 and depicted in figure 6, a 5.7 kb region of cosmid C1A.3.1 was sequenced. This fragment was known to contain the 900 base fragment which expressed P815A in transfectants. This experiment permitted delineation of introns and exons, since the cosmid is genomic in origin.

The delineated structure of the gene is shown in figure 5. Together with figure 6, these data show that the gene for the antigen, referred to as "P1A" hereafter, is approximately 5 kilobases long and contains 3 exons. An orf for a protein of 224 amino acids starts in exon 1, ending in exon 2. The 900 base pair fragment which transfers expression of antigens A and B only contains exon 1. The promoter region contains a CAAT box, as indicated in figure 6a, and an enhancer sequence. This latter feature has been observed in promoters of most MHC class I genes, as observed by Geraghty et al., J. Exp. Med 171: 1-18 (1990); Kimura et al., Cell 44: 261-272 (1986).

A computer homology search was carried out, using program FASTA with K-triple parameters of 3 and 6, as suggested by Lipman et al., Science 227: 1435-1441 (1985), and using Genbank database release 65 (October 1990). No homology was found except for a stretch of 95 bases corresponding to part of an acid region coded by exon 1 (positions 524-618), which is similar to sequences coding for acidic regions in mouse nucleolar protein NO38/B23, as described by Bourbon et al., Mol. Biol. 200: 627-638 (1988), and

Schmidt-Zachmann et al., Chromosoma 96: 417-426 (1988). Fifty six of 95 bases were identical. In order to test whether these homologies were the reason for cross hybridizing, experiments were carried out using a mouse spleen cDNA library screened with the 900 base fragment. cDNA clones corresponding closely to the sizes of the cross hybridizing bands were obtained. These were partially sequenced, and the 2.6 kb cDNA was found to correspond exactly to reported cDNA sequence of mouse nucleolin, while the 1.5 kb cDNA corresponded to mouse nucleolar protein NO38/B23.

Analysis of the nucleotide sequence of the gene, referred to as "P1A" hereafter, suggests that its coded product has a molecular mass of 25 kd. Analysis of the sequence of figure 6 and sequence id no: 1 shows a potential nuclear targeting signal at residues 5-9 (Dingwall et al., Ann. Rev. Cell Biol. 2: 367-390 (1986)), as well as a large acidic domain at positions 83-118. As indicated supra, this contains the region of homology between P1A and the two nucleolar proteins. A putative phosphorylation site can be found at position 125 (serine). Also, a second acidic domain is found close to the C-terminus, and uninterrupted stretch of 14 glutamate residues. A similar C-terminal structure has been found by Kessel et al. Proc. Natl. Acad. Sci. USA 84: 5306-5310 (1987), in a murine heterodomain protein having nuclear localization.

In studies comparing the sequence of gene P1A to the sequences for P91A, 35B and P198, no similarities were found, showing that P1A is indicative of a different class of genes and antigens.

EXAMPLE 10

With the P1A probe and sequence in hand, investigations were carried out to determine whether the gene present in normal tissue was identical to that expressed by the tumor. To do this, phage libraries were prepared, using lambda g 10 and genomic DNA of DBA12 murine kidney cells. As probe, P1A was used. Hybridization conditions were as described supra, and a hybridizing clone was found. The clone contained exons one and two of the P1A gene, and corresponded to positions -0.7 to 3.8 of figure 5. Following localization of this sequence, PCR amplification was carried out to obtain the sequence corresponding to 3.8 to 4.5 of figure 5.

Sequence analysis was carried out, and no differences were found between the gene from normal kidneys and the P1A gene as obtained from the P815 tumor cells.

In further experiments, the gene as found in DBA/2 kidney cells was transfected into PO.HTR, as described supra. These experiments, presented pictorially in figure 7, showed that antigens A and B were expressed as efficiency with the kidney gene as with the P815 gene.

These experiments lead to the conclusion that the gene coding for the tumor rejection antigen is a gene that does not result from a mutation; rather, it would appear that the gene occurs in normal cells. The ramifications of this finding are important, and are discussed infra.

In studies not elaborated upon herein, it was found that variants of the gene were available. Some cells were "P1A^{-}B^{+}", rather than the normal "P1A". The only difference between these is a point mutation in exon 1, with the 18th triplet coding for Ala in the variant instead of Val.

EXAMPLE 11

Additional experiments were carried out with other cell types. Following the protocols described for Northern blot hybridizations supra, RNA of normal liver and spleen cells was tested to determine if a transcript of the P1A gene could be found. The Northern blot data are presented in figure 4 and, as can be seen, there is no evidence of expression.

The murine P815 cell line from which P1A was isolated is a mastocytoma. Therefore, mast cell lines were studied to determine if they expressed the gene. Mast cell line MC/9, described by Nabel et al., Cell 23: 19-28 (1981), and short term cultures of bone marrow derived mast cells were tested in the manner described supra (Northern blotting), but no transcript was found. In contrast when Balb/c derived IL-3 dependent cell line L138.8A (Hültner et al., J. Immunol. 142: 3440-3446 (1989)) was tested, a strong signal was found. The mast cell work is shown in figure 4.

It is known that both BALB/c and DBA/2 mice share H-2^{d} haplotype, and thus it was possible to test sensitivity to lysis using the CTLs described supra. Figure 8 shows these results,

which essentially prove that anti-A and anti-B CTLs lysed the cells strongly, whereas anti-C and anti-D lines did not.

Further tests were carried out on other murine tumor cell lines, i.e., teratocarcinoma cell line PCC4 (Boon et al., Proc. Natl. Acad. Sci. USA 74: 272-275 (1977), and leukemias LEC and WEH1-3B. All showed no hybridization.

EXAMPLE 12

The actual presentation of the P1A antigen by MHC molecules was of interest. To test this, cosmid C1A.3.1 was transfected into fibroblast cell line DAP, which shows phenotype H-2^k. The cell lines were transfected with genes expressing one of the K^d, D^d, and L^d antigen. Following transfection with both the cosmid and the MHC gene, lysis with CTLs was studied, again as described supra. These studies, summarized in Table 2, show that L^d is required for presentation of the P1A antigens A and B.

Table 2. H-2-restriction of antigens P815A and P815B

Recipient cell*	No. of clones lysed by the CTL/ no. of H-mB⁺ clones*	
	CTL anti-A	CTL anti-B
DAP ($H\text{-}2^k$)	0/208	0/194
DAP + K^d	0/165	0/162
DAP + D^d	0/157	0/129
DAP + L^d	25/33	15/20

*Cosmid C1A.3.1 containing the entire P1A gene was transfected in DAP cells previously transfected with $H\text{-}2^d$ class I genes as indicated.

*Independent drug-resistant colonies were tested for lysis by anti-A or anti-B CTL in a visual assay.

EXAMPLE 13

Using the sequence of the P1A gene as well as the amino acid sequence derivable therefrom, antigenic peptides which were A^+ B^+ (i.e., characteristic of cells which express both the A and B antigens), and those which are A^-B^+ were identified. The peptide is presented in Figure 10. These peptides, when administered to samples of P815 cells in the presence of CTL cell lines, led to lysis of the P815 cells, lending support to the view that peptides based on the gene can be used as vaccines.

The melanoma cell line referred to hereafter as MZ2-MEL is not a clonal cell line. It expresses four stable antigens recognized by autologous CTLs, known as antigens "D, E, F, and A". In addition, two other antigens "B" and "C" are expressed by some sublines of the tumor. CTL clones specific for these six antigens are described by Van den Eynde et al., Int. J. Canc. 44: 634-640 (1989). Among the recognized subclones of MZ2-MEL are MEL.43, MEL3.0 and MEL3.1. (Van den Eynde et al., supra). Cell line MEL3.1 expresses antigen E, as determined by CTL studies as described for P815 variants, supra, so it was chosen as a source for the nucleic acid sequence coding the antigen.

In isolating the pertinent nucleic acid sequence for a tumor rejection antigen, the techniques developed supra, showed that a recipient cell is needed which fulfills two criteria: (i) the recipient cell must not express the TRA of interest under normal conditions, and (ii) it must express the relevant class I HLA molecule. Also, the recipient cell must have a high transfection frequency, i.e., it must be a "good" recipient.

In order to secure such a cell line, the clonal subline ME3.1 was subjected to repeated selection with anti-E CTL 82/30 as described by Van den Eynde, supra. The repeated cycles of selection led to isolation of subclone MZ2-MEL-2.2 isc E^-. This subclone is also $HPRT^-$, (i.e., sensitive to HAT medium: 10^{-4} M hypoxanthine, 3.8×10^{-7} aminopterine, 1.6×10^{-5} M 2-deoxy-

thymidine). The subclone is referred to as "MEL-2.2" for simplicity hereafter.

EXAMPLE 14

The genomic DNA of MEL3.0 was prepared following Wölfel et al., Immunogenetics 26: 178-187 (1987), the disclosure of which is incorporated by reference. The plasmid pSVtkneoß, as described by Nicolas et al., Cold Spring Harb., Conf. Cell Prolif. 10: 469-485 (1983) confers geneticin resistancee, so it can be used as a marker for cotransfection, as it was in this experiment.

Following a procedure similar but not identical to that of Corsao et al., Somatic Cell Molec. Genet 7: 603-616 (1981), total genomic DNA and the plasmid were cotransfected. The genomic DNA (60 μg) and plasmid DNA (6 μg) were mixed in 940 μl of 1 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, after which 310 μl of 1M $CaCl_2$ was added. This solution was slowly added, under constant agitation, to 1.25 ml of 2xHBS (50 mM HEPES, 280 mM NaCl 1.5 mM Na_2HPO_4, adjusted to pH 7.1 with NaOH). The calcium phosphate DNA precipitates were allowed to form for 30-45 minutes at room temperature, after which they were applied to 80 cm^2 tissue culture flasks which had been seeded 24 hours previously with $3x10^6$ MEL2.2 cells, in 22.5 ml of melanoma culture medium (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal calf serum. After 24 hours, the medium was replaced. Forty eight hours after transfection, the cells were harvested and seeded at $4x10^6$ cells

per 80 cm^2 flask in melanoma culture medium supplemented with 2 mg/ml of geneticin. The geneticin serves as a selection marker.

EXAMPLE 15

Thirteen days after transfection, geneticin-resistant colonies were counted, harvested, and cultured in nonselective medium for 2 or 3 days. Transfected cells were then plated in 96-well microplates at 200 cells/well in 200 ul of culture medium with 20% fetal calf serum (FCS) in order to obtain approximately 30 growing colonies per well. The number of microcultures was aimed at achieving redundancy, i.e., such that every independent transfectant should be represented at least four times.

After 10 days, wells contained approximately $6x10^4$ cells. These cells were detached, and 1/3 of each microculture was transferred to a duplicate plate. After 6 hours, i.e., after readherence, medium was removed and 1500 anti-E CTL (CTL 82/30), were added to each well in 100 μl of CTL culture medium with 35 U/ml of IL-2. One day later, the supernatant (50 μl) was harvested and examined for TNF concentration, for reasons set forth in the following example.

EXAMPLE 16

The size of the mammalian genome is $6x10^6$ kb. As the average amount of DNA integrated in each drug-resistant transfectant was expected to be about 200 kb, a minimum of 30,000 transfectants

would need to be examined to ascertain whether antigen E had been transfected. Prior work with murine cells had shown that when a CTL stimulation assay was used, groups containing only 3% of cells expressing the antigen of interested could be identified. This should reduce the number of assays by a factor of 30. While an anti-E CTL assay, as described supra, in mixed E^+/E^- cells was helpful, it was not sufficient in that consistent results could not be obtained.

As a result, an alternative test was devised. Stimulation of CTLs was studied by release of tumor necrosis factor ("TNF"). As described in Example 15, 1500 CTL 82/30 cells had been added per well of transfectants. These cells were collected 6 days after stimulation, and were then restimulated for 24 hours in a mixture of 40,000 E^+ and E^- cells in mixed culture. As indicated supra, after 1/3 of the cells in each well had been removed and the remaining 2/3 ($4x10^4$) had readhered, the CTLs and IL-2 were added thereto. The 50 μl of supernatant was removed and transferred to a microplate containing $3x10^4$ W13 (WEHI-164 clone 13) cells in 50 μl of W13 culture medium (RPMI-1640, supplemented with L-arginine (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), and 10% FCS) supplemented with 2 μg of actinomycin D at 37% in an 8% CO_2 atmosphere. The cell line W13 is a mouse fibrosarcoma cell line sensitive to TNF. Dilutions of recombinant TNF-ß in RPMI 1640 were added to target cell controls.

The W13 cultures were evaluated after 20 hours of incubation, and dead cell percentage was measured using an adaptation of the colorimetric assay of Hansen et al., J. Immunol. Meth. 119: 203-210 (1989). This involved adding 50 ml of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide at 2.5 mg/ml in PBS, followed by two hours of incubation at 37°C. Dark blue formazan crystals were dissolved by adding 100 µl of lysis solution (1 volume N,N dimethyl formamide mixed at 37°C with two volumes of water containing 30% (w/v) sodium dodecyl sulphate, at pH 47 from 1.6% acetic acid and 2.5% 1N HCl). Plates were incubated at 37°C overnight, and ODs were taken at 570 nm using 650 nm as control. Dead cell percentage was determined via the formula:

$$100 \times \left[1 - \frac{100-(OD_{570} \text{ sample well})}{OD_{570} \text{ well + medium}} \right]$$

following Espevik et al., J. Immunol. Meth. 95: 99-105 (1986). The results showed that even when the ratio of E^+/E^- cells was as low as 1/45, significant production of TNF was observed, thus showing active CTLs. This led to the decision to test the drug resistant transfectants in groups of 30.

EXAMPLE 17

Cells were tested for TNF production as discussed in Example 16, supra. A total of 100 groups of E^- cells ($4x10^6$ cells/group) were tested following transfection, and $7x10^4$ independent geneticin

resistant transfectants were obtained, for an average of 700 per group. Only one group of transfected cells led to a microculture which caused anti-E antigen CTL clone 82/30 to produce TNF. Of 300 clones tested, 8 were positive. These clones were then tested for lysis by anti-E CTL, using the standard ^{51}Cr release assay, and were found to be lysed as efficiently as the original E^+ cell line. The transfectant E.T1, discussed herein, had the same lysis pattern as did MEL2.2 for CTLs against antigens B,C,D and F.

The fact that only one transfectant expressed the antigen out of 70,000 geneticin resistance transfectants may at first seem very low, but it is not. The work described supra for P815 showed an average frequency of 1/13,000. Human DNA recipient MEL2.2 appears to integrate 5 times less DNA than P1.HTR.

EXAMPLE 18

Once transfectant E.T1 was found, analysis had to address several questions including whether an E^+ contaminant of the cell population was the cause. The analysis of antigen presentation, described supra, shows that E.T1 is B^- and C^-, just like the recipient cell MEL2.2. It was also found to be $HPRT^-$, using standard selection procedures. All E^+ cells used in the work described herein, however, were $HPRT^+$.

It was also possible that an E^+ revertant of MEL2.2 was the source for E.T1. To test this, the observation by Perucho et al., Cell 22: 309-317 (1980), that cotransfected sequences usually

integrate together at a single location of recipient genome was employed. If antigen E in a transfectant results from cotransfection with pSVtkneoß, then sequences should be linked and deletion of the antigen might also delete the neighboring pSVtkneoß sequences. Wölfel et al., supra, has shown this to be true. If a normally E^- cell transfected with pSVtkneoß, then sequences should be linked and deletion of the antigen might also delete the neighboring pSVtkneoß sequences. Wölfel et al., supra, has shown this to be true. If a normally E^+ cell transfected with pSVtkneoß is E.T1, however, "co-deletion" should not take place. To test this, the transfectant E.T1 was subjected to immunoselection with 82/30, as described supra. Two antigen loss variants were obtained, which resisted lysis by this CTL. Neither of these had lost geneticin resistance; however, Southern blot analysis showed loss of several neo^r sequences in the variants, showing close linkage between the E gene and neo^r gene in E.T1, leading to the conclusion that E.T1 was a transfectant.

EXAMPLE 20

The transfectant E.T1 was used as a source of DNA for preparation of a cosmid library. This library of nearly 50,000 cosmids was transfected into MZ2-MEL 2.2 cells, following the cosmid transfection protocols described supra. One cosmid, referred to as B3, was recovered from this experiment, and subjected to restriction endonuclease digestion via SmaI, or by

BamHI digestion of a large, 12 kb SmaI transfected fragment. The fragments were cloned into vector pTZ 18, and then into MEL2.2. Again, TNF production was the measure by which successful transfection was determined. The experiments led to the determination of a gene sequence capable of transfecting antigen E on the 12 kb SmaI fragment, and then on the 2.4 kb fragment of BamHI digestion of the 12 kb segment.

The 2.4 kb fragment hybridizes with a 2.4 kb fragment from MZ2-MEL and with a T cell clone of patient MZ-2, as determined by Southern Blots. The band is absent from E^- antigen loss variants of MZ2-MEL.

The sequence for the E antigen gene has been determined, and is presented herein:

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        |  10      |  20      |  30      |  40      |  50      |  60
   1 GGATCCAGGC CCTGCCAGGA AAAATATAAG GGCCCTGCGT GAGAACAGAG GGGGTCATCC 60
  61 ACTGCATGAG AGTGGGGATG TCACAGAGTC CAGCCCACCC TCCTGGTAGC ACTGAGAAGC 120
 121 CAGGGCTGTG CTTGCGGTCT GCACCCTGAG GGCCCGTGGA TTCCTCTTCC TGGAGCTCCA 180
 181 GGAACCAGGC AGTGAGGCCT TGGTCTGAGA CAGTATCCTC AGGTCACAGA GCAGAGGATG 240
 241 CACAGGGTGT GCCAGCAGTG AATGTTTGCC CTGAATGCAC ACCAAGGGCC CCACCTGCCA 300
 301 CAGGACACAT AGGACTCCAC AGAGTCTGGC CTCACCTCCC TACTGTCAGT CCTGTAGAAT 360
 361 CGACCTCTGC TGGCCGGCTG TACCCTGAGT ACCCTCTCAC TTCCTCCTTC AGGTTTTCAG 420
 421 GGGACAGGCC AACCCAGAGG ACAGGATTCC CTGGAGGCCA CAGAGGAGCA CCAAGGAGAA 480
 481 GATCTGTAAG TAGGCCTTTG TTAGAGTCTC CAAGGTTCAG TTCTCAGCTG AGGCCTCTCA 540
 541 CACACTCCCT CTCTCCCCAG GCCTGTGGGT CTTCATTGCC CAGCTCCTGC CCACACTCCT 600
 601 GCCTGCTGCC CTGACGAGAG TCATCATGTC TCTTGAGCAG AGGAGTCTGC ACTGCAAGCC 660
 661 TGAGGAAGCC CTTGAGGCCC AACAAGAGGC CCTGGGCTGG TGTGTGTGCA GGCTGCCACC 720
 721 TCCTCCTCCT CTCCTCTGGT CCTGGGCACC CTGGAGGAGG TGCCCACTGC TGGGTCAACA 780
 781 GATCCTCCCC AGAGTCCTCA GGGAGCCTCC GCCTTTCCCA CTACCATCAA CTTCACTCGA 840
 841 CAGAGGCAAC CCAGTGAGGG TTCCAGCAGC CGTGAAGAGG AGGGGCCAAG CACCTCTTGT 900
 901 ATCCTGGAGT CCTTGTTCCG AGCAGTAATC ACTAAGAAGG TGGCTGATTT GGTTGGTTTT 960
 961 CTGCTCCTCA AATATCGAGC CAGGGAGCCA GTCACAAAGG CAGAAATGCT GGAGAGTGTC 1020
1021 ATCAAAAATT ACAAGCACTG TTTTCCTGAG ATCTTCGGCA AAGCCTCTGA GTCCTTGCAG 1080
1081 CTGGTCTTTG GCATTGACGT GAAGGAAGCA GACCCCACCG GCCACTCCTA TGTCCTTGTC 1140
1141 ACCTGCCTAG GTCTCTCCTA TGATGGCCTG CTGGGTGATA ATCAGATCAT GCCCAAGACA 1200
1201 GGCTTCCTGA TAATTGTCCT GGTCATGATT GCAATGGAGG GCGGCCATGC TCCTGAGGAG 1260
1261 GAAATCTGGG AGGAGCTGAG TGTGATGGAG GTGTATGATG GGAGGGAGCA CAGTGCCTAT 1320
1321 GGGGAGCCCA GGAAGCTGCT CACCCAAGAT TTGGTGCAGG AAAAGTACCT GGAGTACGGC 1380
1381 AGGTGCCGGA CAGTGATCCC GCACGCTATG AGTTCCTGTG GGGTCCAAGG GCCCTCGCTG 1440
1441 AAACCAGCTA TGTGAAAGTC CTTGAGTATG TGATCAAGGT CAGTGCAAGA GTTCGCTTTT 1500
1501 TCTTCCCATC CCTGCGTGAA GCAGCTTTGA GAGAGGAGGA AGAGGGAGTC TGAGCATGAG 1560
1561 TTGCAGCCAA GGCCAGTGGG AGGGGGACTG GGCCAGTGCA CCTTCCAGGG CCGCGTCCAG 1620
1621 CAGCTTCCCC TGCCTCGTGT GACATGAGGC CCATTCTTCA CTCTGAAGAG AGCGGTCAGT 1680
1681 GTTCTCAGTA GTAGGTTTCT GTTCTATTGG GTGACTTGGA GATTTATCTT TGTTCTCTTT 1740
1741 TGGAATTGTT CAAATGTTTT TTTTTAAGGG ATGGTTGAAT GAACTTCAGC ATCCAAGTTT 1800
1801 ATGAATGACA GCAGTCACAC AGTTCTGTGT ATATAGTTTA AGGGTAAGAG TCTTGTGTTT 1860
1861 TATTCAGATT GGGAAATCCA TTCTATTTTG TGAATTGGGA TAATAACAGC AGTGGAATAA 1920
1921 GTACTTAGAA ATGTGAAAAA TGAGCAGTAA AATAGATGAG ATAAAGAACT AAAGAAATTA 1980
1981 AGAGATAGTC AATTCTTGCC TTATACCTCA GTCTATTCTG TAAAATTTTT AAAGATATAT 2040
2041 GCATACCTGG ATTTCCTTGG CTTCTTTGAG AATGTAAGAG AAATTAAATC TGAATAAAGA 2100
2101 ATTCTTCCTG TTCACTGGCT CTTTTCTTCT CCATGCACTG AGCATCTGCT TTTTGGAAGG 2160
2161 CCCTGGGTTA GTAGTGGAGA TGCTAAGGTA AGCCAGACTC ATACCCACCC ATAGGGTCGT 2220
2221 AGAGTCTAGG AGCTGCAGTC ACGTAATCGA GGTGGCAAGA TGTCCTCTAA AGATGTAGGG 2280
2281 AAAAGTGAGA GAGGGGTGAG GGTGTGGGGC TCCGGGTGAG AGTGGTGGAG TGTCAATGCC 2340
2341 CTGAGCTGGG GCATTTTGGG CTTTGGGAAA CTGCAGTTCC TTCTGGGGGA GCTGATTGTA 2400
2401 ATGATCTTGG GTGGATCC                                              2418
        |  10      |  20      |  30      |  40      |  50      |  60
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The approach taken in the isolation and study of the tumor rejection antigen genes P1A and E can be correlated with studies reported by several authors (reviewed by Anichini et al., Immunol. Today 8: 385-389 (1987)), and including Hérin et al., Int. J. Canc. 39: 390-396 (1987), who report that blood lymphocytes of many human cancer patients can be restimulated in vitro with their own tumor cells, so as to produce CTLs that lyse cancer cells with specificity and particularity. Such an approach, coupled with the observations described herein, suggests a method for developing a therapeutic approach to cancer. In such a method, the tumor rejection antigen gene of interest is isolated and transfected into a recipient cell. The transfectant is then introduced or contacted to the lymphocytes of the patient so as to generate cytotoxic T cells specific to the tumor from which the gene was isolated. Either the mixed cultures of CTLs, or specific cell lines can be introduced to the subject or patient, so as to provoke lysis of the tumor cells of the subject.

Transfection of a host cell with a recognized tumor rejection antigen may also permit in vitro diagnosis of patients who may be in the process of developing tumors. Specifically, since it has been shown that tumor rejection antigens do provoke an immune response, via T cells, in vivo, diagnosis of a patient can take place by contacting a lymphocyte containing patient sample to transfectants and observing lysis of the transfectants as an indication of the presence of appropriate CTLs. This methodology may also facilitate pathological diagnosis of tumors, as it is

reasonable to conclude that related tumors will share the relevant antigen of class described supra.

It is also within the invention to contemplate the use of the tumor rejection antigen or fragments thereof as vaccines to prevent the onset or re-occurrence of cancer. This is particularly desirable in the case of individuals in high risk families, genetic predisposition to development of cancer being well accepted by the art.

One aspect of the invention is the ability to prepare whole cell vaccines for patients, based upon the invention. In brief, it is now recognized by the inventors that genes can be identified and isolated which are characteristic of a particular type of cancer, e.g., melanoma. It is expected that families of these TRA genes will be identified for each cancer or tumor type.

With these families of genes identified, one then works with a sample of a tumor from a patient. Isolation of RNA from the tumor cells can be carried out, using techniques well known in the art. This total RNA can then be probed with the various DNA sequences known to be representative of the tumor type. This can be done using any of the standard techniques used for nucleic acid identification and probing, including the polymerase chain reaction ("PCR") technique.

This will allow the investigator to identify which TRA gene is being expressed by the tumor. With this information in hand, the investigator may turn to a "bank" of cell lines expressing the various TRAs to choose one which expresses the various TRAs and an

appropriate HLA molecule which is immunologically identical (i.e., generates an equivalent CTL response) to the patient's tumor. As has been shown, supra, such cell lines generate CTL responses leading to lysis.

Samples of the cell line may be treated to render them incapable of multiplication, such as by irradiation. The non-replicable cells are then introduced to the patient in an amount sufficient to generate a CTL mediated response, leading to lysis of the patient's tumor.

As observed supra, the gene for the P815 tumor rejection antigen is identical to a gene found in normal cells. This raises the question of why an autoimmune response is not generated in individuals, since tumors presenting the antigen clearly do. While not binding themselves to a particular theory, the inventors note several potential hypotheses. The first of these is based on the notion of oncofetal antigens, which implies that tumors re-express antigens which have disappeared from normal cells before immunological tolerance is generated. Thus, normal cell simply do not express the antigen, even though the gene is present. Alternately, the gene may be expressed by a very small number of mast cell precursors during a very brief stage of differentiation. Either because of small numbers or wide dispersion or both, or immune response is simply not established.

The invention therefore describes isolated and purified DNA sequences which code for tumor rejection antigens, and the tumor rejection antigen coded for by the DNA sequences. Also described

are transfected cells which contain the isolated DNA sequences, as well as methods for use of the gene and the antigen for diagnostic and therapeutic uses explained supra.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.